bio-protocol Preprint

Not Peer Reviewed
Ask a question online

Drosha cleavage assay

Tsui Han Loo

Updated date: Dec 6, 2019

An abbreviated version of this protocol was published in eLIFE in Nov 2019

The mammalian LINC complex component SUN1 regulates muscle regeneration by modulating drosha activity

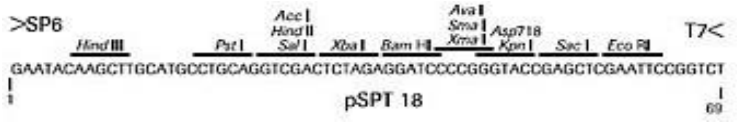
DOI: 10.7554/eLife.49485

Detailed protocol

Treat gel tank/apparatus with 0.1% DEPC for RNA work.

Synthesize RNA substrate for Drosha cleavage assay

Clone pre-miR127 with flanking 100bp (total 269bp) into pSPT18 vector via HindIII/EcoRI restriction sites, to minimise sequence from pSPT18 vector in the final RNA transcript



Digest 5ug of pSPT18 pri-miR127 with EcoRI at 37^{O}C overnight, resolve DNA with agarose gel to make sure **complete digestion**, excise band and purify DNA (Qiagen QIAquick PCR Purification Kit) for SP6 transcription

Perform in vitro transcription reaction fresh for Drosha cleavage assay

Use 400ng digested DNA with (Roche or Promega) SP6 in vitro transcription in final 100ul reaction, 37^{O}C for 2hr, then treat with DNase for 15min

10ul is enough for 1 Drosha cleavage reaction

For checking

The synthesis of the pri-miR127 RNA can be check by DIG-UTP labelling, load 1ul of the transcription rxn (with Novex TBE urea sample buffer) onto 6% Novex TBE-urea gel and run for 180V for 90min

Wet transfer to Hybond membrane with 0.5x TBE buffer 30V for 1 hr

UV-cross link, block with Roche 20% blocking reagent (Cat 11585762001) and anti-DIG AP @ 1/10,000 and CDP-Star to develop

- For Drosha cleavage assay, synthesis RNA with regular rNTPs, no DIG labelling.

Detecting Drosha cleaved RNA (pre-miRNA) with DIG-labelled probe

Clone pre-miR127 (67nt) into pSPT18 vector via HindIII/EcoRI

Digest pSPT18 pre-miR127 with HindIII at 37^{O}C overnight, resolve DNA with agarose gel to make sure complete digestion, excise band and purify for T7 transcription

Use 400ng digested DNA with (Promega or Roche T7) UDP/ DIG-UTP transcription, 37^{O}C for 2hr, then treat with DNase for 15min

Producing Pasha /Drosha protein complex for RNA cleavage assay

Transient transfect Pasha and Drosha (1ug & 2ug DNA respectively) with Lipofectamine 2000 in T25 flask of HEK293 cells for 24hours

Cell lysis in 500ul buffer and sonication, immunoprecipitate with M2 beads (Pasha and Drosha are Flag tagged), wash and equilibrate with buffer containing 10mM Tris pH7.4 and 10mM MgCl2

Add buffer (total 30ul) containing RNase inhibitor, pri-miR RNA to M2 beads-Drosha/Pasha protein complex and incubate at 37^{O}C for 90min

Add Novex TBE urea sample buffer to the above and heat at 70^{O}C for 3min, load onto 6% TBE-Urea gel and run for 180V for 90min.

Wet transfer to Hybond membrane with 0.5x TBE buffer 30V for 1 hr, and then UV cross-link

Add DIG-labelled RNA probe in hybridisation buffer (Roche Northern kit) to Hybond membrane, and incubate at 42^{O}C for overnight rolling,

Hybond membrane wash with SSC (decreasing strength 5x, 2x, 0.1x) and detection with anti-DIG AP and CDP-Star to develop

Reagents

Novex TBE urea sample buffer 2x (LC6876)

Novex 6% TBE urea gel (EC68652)

Roche Anti-DIG-AP Fab fragment (11093274910)

Roche DIG Northern Starter Kit (12039672910)

Amersham Hybond TM-N+

How to cite:(Readers should cite both the Bio-protocol preprint and the original research article where this protocol was used)

1. Loo, T. H.(2019). Drosha cleavage assay. Bio-protocol Preprint. bio-protocol.org/prep99.
2. Loo, T. H., Ye, X., Chai, R. J., Ito, M., Bonne, G., Ferguson-Smith, A. C. and Stewart, C. L.(2019). The mammalian LINC complex component SUN1 regulates muscle regeneration by modulating drosha activity. eLIFE. DOI: 10.7554/eLife.49485